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**13. ABSTRACT** ***(Maximum 200 Words)***

ErbB4 is a member of the signaling network composed of the EGF (epidermal growth factor) family of peptide hormones and the ErbB family of receptor tyrosine kinases. We have hypothesized that ErbB4 is a mammary-specific tumor suppressor. Indeed, we have preliminary data that indicates that overexpression of ErbB4 causes the EGF family hormones EGF and Neuregulin to inhibit DNA synthesis of a human mammary tumor cell line. Furthermore, we have constructed three constitutively active, ligand-independent ErbB4 mutants. We have published data indicating that these mutants do not malignantly transform the growth of cultured rodent fibroblasts. These data indicate that ErbB4 is not an oncogene. Moreover, we have preliminary data indicating that one of the three constitutively active ErbB4 mutants inhibits proliferation of at least one human mammary cell line. Future experiments are planned to examine whether this response to ErbB4 signaling is observed in a panel of human mammary cell lines. Other experiments are planned to begin to elucidate the signaling transduction pathway that couples ErbB4 to inhibition of mammary cell proliferation.

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## Table of Contents

## Introduction

ErbB4 is a member of the signaling network composed of the EGF (epidermal growth factor) family of peptide hormones and the ErbB family of receptor tyrosine kinases. We have hypothesized that ErbB4 is a mammary-specific tumor suppressor. Indeed, we have preliminary data that indicate that overexpression of ErbB4 causes the EGF family hormones EGF and Neuregulin to inhibit DNA synthesis of a human mammary tumor cell line.

This IDEA award is partially supporting our efforts to elucidate the role of ErbB4 in mammary tumorigenesis. Our goals are to (1) generate a constitutively-active ErbB4 mutant; (2) assess whether the constitutively-active ErbB4 mutant inhibits the proliferation of human mammary cell lines; (3) assess whether the constitutively-active ErbB4 mutant malignantly transforms the growth of human mammary cell lines; (4) generate ErbB4 double mutants in the context of the constitutively active mutant that are either deficient for kinase activity or contain mutations at tyrosine phosphorylation sites; (5) assess whether ErbB4 kinase activity or specific ErbB4 tyrosine phosphorylation sites are critical for coupling to biological activity.

## Report Body

*1. Generate a constitutively active ErbB4 mutant.* The major goals of the proposed research are to generate a constitutively active ErbB4 mutant and to use this mutant to probe ErbB4 function. We attempted to construct a constitutively active ErbB4 mutant by deleting the extracellular ligand-binding domain. However, we could not stably express this ErbB4 mutant. We attempted to construct a constitutively active ErbB4 mutant by replacing the transmembrane domain of human wild-type ErbB4 with the transmembrane domain of a constitutively active rat ErbB2/HER2/Neu mutant. However, we could not stably express this ErbB2-ErbB4 chimeric protein.

Our third attempt to generate a constitutively active ErbB4 mutant involved introducing single cysteine substitutions at five different locations in the ErbB4 extracellular juxtamembrane domain. Of these five ErbB4 mutants, three (Q646C, H647C, A648C) appear to be constitutively active for signaling; they exhibit greater kinase activity in the absence of ligand than does wild-type ErbB4 (Figure 1) and they exhibit more tyrosine phosphorylation in the absence of ligand than does wild-type ErbB4 (Figure 2). These data are taken from a paper recently published by my laboratory in *Cell Growth and Differentiation* (Penington, *et al.*) A reprint of this paper appears in the appendix.

These mutants do not malignantly transform the growth of rodent fibroblast cell lines. They do not induce growth in semi-solid medium in an assay for anchorage independence (Figure 3). They do not stimulate the growth rate or saturation density (Figure 4). Finally, they do not induce foci in an assay for loss of contact inhibition (Figure 5). These data are taken from a paper recently published by my laboratory in *Cell Growth and Differentiation* (Penington, *et al.*) A reprint of this paper appears in the appendix.

*2. Assess whether the constitutively active ErbB4 mutant inhibits the proliferation of human mammary cell lines.* We have preliminary data that indicate that ErbB4 overexpression in the SKBR3 human mammary tumor cell line causes the EGF family hormones EGF and NRG1β to inhibit cellular DNA synthesis (Figure 6). This suggests that ErbB4 is coupled to growth arrest and that ErbB4 may be a tumor suppressor.

We have investigated this hypothesis further by infecting MCF-10A human mammary epithelial cells with recombinant retroviruses that express the constitutively active ErbB4 mutants as well as a selectable marker (the neomycin resistance gene, which confers resistance to the antibiotic G418). We are also infecting cells with control recombinant retroviruses that contain the neomycin resistance gene alone (vector control), or the neomycin resistance gene along with wild-type ErbB4 or a constitutively active ErbB2 mutant.

MCF-10A cells infected with the recombinant retrovirus that carries the Q646C ErbB4 mutant form far fewer drug-resistant colonies than MCF-10A cells infected with the other retroviruses (Figure 7). We infected C127 cells with the various recombinant retroviruses and quantified the number of drug-resistant colonies that resulted to permit us to account for possible differences in absolute viral titer. Even after accounting for differences in absolute viral titer, infection of MCF-10A cells with the retrovirus that contains the Q646C ErbB4 mutant results in far fewer drug-resistant colonies than expected (data not shown). This suggests that the Q646C constitutively active ErbB4 mutant is coupled to inhibition of proliferation or induction of apoptosis in MCF-10A cells.

We have performed analogous preliminary experiments with the DU-145, PC-3 and LNCaP human prostate tumor cell lines (Figures 8-10). In all three cell lines the Q646C ErbB4

mutant inhibits formation of drug-resistant colonies in plastic dishes. We are currently performing analogous experiments with a panel of human mammary tumor cell lines (MCF-7, MDA-MB-231, MDA-MB-453, SKBR-3, and T47-D) to determine whether the Q646C ErbB4 mutant inhibits drug-resistant colony formation in these cells as well.

Finally, we have infected a panel of human mammary (tumor) cell lines (MCF-7, MCF-10A, MDA-MB-231, MDA-MB-453, SKBR-3, and T47-D) with the various recombinant retroviruses and generated stable cell lines. We had hoped to assess whether the Q646C constitutively active ErbB4 mutant or either of the other two constitutively active ErbB4 mutants affects mammary cell growth rates or saturation densities. Unfortunately, very few drug-resistant colonies arose following infection with the recombinant retrovirus that carries the Q646C ErbB4 mutant. Furthermore, those colonies that did arise failed to ectopically express wild type ErbB4 (data not shown).

*3. Assess whether the constitutively active ErbB4 mutant malignantly transforms the growth of human mammary cell lines.* The constitutively active ErbB4 mutants fail to malignantly transform the growth of fibroblasts. Thus, we believe it is unlikely that the constitutively active ErbB4 mutants will stimulate or increase anchorage-independent growth human mammary tumor cell lines. Furthermore, we are unable to stably express the Q646C ErbB4 mutant in human mammary cell lines. Consequently, we have decided not to pursue these experiments.

*4. Generate ErbB4 double mutants in the context of the constitutively active mutant that are either deficient for kinase activity or contain mutations at tyrosine phosphorylation sites.* We have constructed a kinase-deficient version of the constitutively active ErbB4 Q646C mutant. We have also created a version of the constitutively active ErbB4 Q646C mutant in which all eight of the putative tyrosine phosphorylation sites have been mutated to phenylalanine. We are currently in the process of creating eight different versions of the constitutively active ErbB4 Q646C mutant in which but a single putative site of tyrosine phosphorylation is present.

*5. Using the ErbB4 double mutants, assess whether ErbB4 kinase activity or specific ErbB4 tyrosine phosphorylation sites are critical for coupling to biological activity.* Preliminary data obtain using the DU-145 and PC-3 prostate tumor cell lines indicate that the kinase-defective version of the constitutively active ErbB4 Q646C mutant is unable to inhibit drug-resistant colony formation on plastic. This suggests that ErbB4 kinase activity is required for the ErbB4 Q646C mutant to couple to inhibition of colony formation. We intend to perform analogous experiments using a panel of human mammary tumor cell lines. Once the ErbB4 Q646C tyrosine phosphorylation site mutants are available, we will test these mutants as well.

## Key Research Accomplishments

*Task 1*

- Generated a number of putative constitutively active ErbB4 mutants.
- Identified three ErbB4 mutants that exhibit ligand-independent tyrosine phosphorylation and increased tyrosine kinase activity.
- Determined that the three constitutively active ErbB4 mutants do not couple to malignant growth transformation in fibroblast cell lines.

*Task 2*

- Generated preliminary data suggesting that the constitutively active Q646C ErbB4 mutant inhibits drug-resistant colony formation by MCF-10A human mammary epithelial cells. Analogous experiments using a panel of human breast tumor cell lines are underway.
- Generated preliminary data suggesting that the constitutively active Q646C ErbB4 mutant inhibits drug-resistant colony formation by a panel of human prostate tumor cell lines.
- Tried to generate a panel of human mammary (tumor) cell lines that express wild-type ErbB4, the constitutively active ErbB4 mutants, a constitutively active ErbB2 mutant, or the vector control. Demonstrated that the Q646C ErbB4 mutant is not expressed in infected human mammary cell lines.

*Task 3*

- We have decided not to pursue these experiments.

*Task 4*

- Generated a kinase-deficient version of the constitutively active ErbB4 Q646C mutant.
- Generating versions of the constitutively active ErbB4 Q646C that lack all or all but one of the putative sites of ErbB4 tyrosine phosphorylation.

*Task 5*

- Preliminary data indicate that a kinase-deficient version of the constitutively active ErbB4 Q646C mutant fails to inhibit drug-resistant colony formation by a panel of human prostate tumor cell lines. Analogous experiments using a panel of human breast tumor cell lines are underway.
- Analogous experiments analyzing the activity of the ErbB45 Q646C phosphorylation site mutants in a panel of human breast tumor cell lines will commence once the mutants are available.

## Reportable Outcomes

- A manuscript that describes the construction and analysis of our constitutively active ErbB4 mutants has been published in *Cell Growth and Differentiation.* A reprint of this paper appears in the appendix of this report (Penington, *et al*).
- Mr. Desi Penington wrote and successfully defended a master's degree thesis entitled "Construction and analysis of constitutively-active mutants of the ErbB4 receptor tyrosine kinase" that is based on the results of the studies described in Task 2. A copy of Mr. Penington's thesis is included in the appendix of this report. Mr. Penington received his M.S. in August 2001.
- A grant application submitted to the USAMRMC PCRP for additional funding to support our efforts to analyze ErbB4 function in prostate cancer cells was selected for funding (DAMD17-02-1-0130, Dr. David J. Riese II, PI).
- We were awarded an undergraduate research fellowship by the American Association of Colleges of Pharmacy to support our efforts to analyze ErbB4 function in prostate cancer cells (Mr. Eric Williams, PI; Dr. David J. Riese II , mentor).
- We were awarded an undergraduate research fellowship by the American Society for Microbiology to support our efforts to analyze ErbB4 function in breast and prostate cancer cells (Ms. Ianthe Bryant, PI; Dr. David J. Riese II, mentor).

## Conclusions

We have made significant progress on the proposed research plan. We have generated three constitutively active ErbB4 mutants. These mutants do not malignantly transform the growth of fibroblasts. Indeed, we have preliminary evidence that one of these mutants is coupled to inhibition of drug-resistant colony formation in a breast cell line. This suggests that ErbB4 may act as a tumor suppressor gene in the mammary epithelium. Furthermore experiments are underway to test this hypothesis. Experiments are also underway to genetically identify the biochemical functions of ErbB4 that are required to couple this receptor to biological responses.

## References

Penington, D.J., I. Bryant, and D.J. Riese II. "Constitutively active ErbB4 and ErbB2 mutants exhibit distinct biological activities." *Cell Growth Diff.* **13**: 247-256 (2002).

## Appendices: List of Documents (20 pages total)

Figure 1. *Q646C, H647C, and A648C ErbB4 mutants exhibit increased kinase activity.* From Penington, *et al., Cell Growth Diff.* **13**: 247-256 (2002).

Figure 2. *Q646C, H647C, and A648C mutants exhibit increased ligand-independent tyrosine phosphorylation.* From Penington, *et al., Cell Growth Diff.* **13**: 247-256 (2002).

Figure 3. *Constitutively active ErbB4 mutants do not cause anchorage independence in FR3T3 fibroblasts.* From Penington, *et al., Cell Growth Diff.* **13**: 247-256 (2002).

Figure 4. *Constitutively active ErbB4 mutants do not cause an increase in growth rate or an increase in saturation density in fibroblasts.* From Penington, *et al., Cell Growth Diff.* **13**: 247-256 (2002).

Figure 5. *Constitutively active ErbB4 mutants do not cause a loss of contact inhibition (focus formation) in FR3T3 fibroblasts.* From Penington, *et al., Cell Growth Diff.* **13**: 247-256 (2002).

Figure 6. *Overexpression of ErbB4 in the SKBR3 human breast tumor cell line causes inhibition of DNA synthesis by EGF and NRG.* Unpublished data.

Figure 7. *The constitutively active Q646C ErbB4 mutant inhibits colony formation by the MCF10A human mammary epithelial cell line.* Unpublished data.

Figure 8. *The constitutively active Q646C ErbB4 mutant inhibits colony formation by the DU145 human prostate tumor cell line.* Unpublished data.

Figure 9. *The constitutively active Q646C ErbB4 mutant inhibits colony formation by the PC-3 human prostate tumor cell line.* Unpublished data.

Figure 10. *The constitutively active Q646C ErbB4 mutant specifically inhibits colony formation by the PC-3, DU-145, and LNCaP human prostate tumor cell lines.* Unpublished data.

Journal Article (10 pages). Penington, D.J., I. Bryant, and D.J. Riese II. "Constitutively active ErbB4 and ErbB2 mutants exhibit distinct biological activities." *Cell Growth Diff.* **13**: 247-256 (2002).

Figure 1. *Q646C, H647C, and A648C ErbB4 mutants exhibit increased kinase activity.*

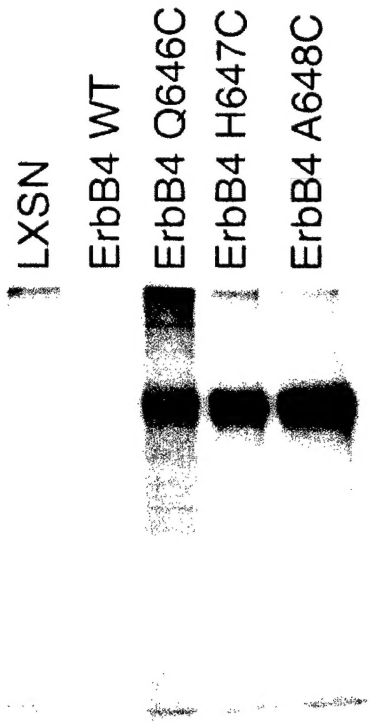



*Fig. 2.* Q646C, H647C, and A648C mutants exhibit increased *in vitro* kinase activity. Equal amounts of protein lysates (1000 $\mu$g) from PA317 cells that stably express wild-type ErbB4 or the ErbB4 mutants (Q646C, H647C, and A648C) were immunoprecipitated with an anti-ErbB4 rabbit polyclonal antibody. Lysates from PA317 cells that express the LXSN vector served as the negative control. Kinase reactions were performed on the immunoprecipitates in the presence of [$\gamma$-$^{32}$P]ATP. The products were resolved by SDS-PAGE. The gel was dried overnight and exposed to X-ray film for ~20 h to visualize the products of the kinase reactions.

From Penington, *et al.*, *Cell Growth Diff.* **13**: 247-256 (2002).

Figure 2. *Q646C, H647C, and A648C mutants exhibit increased ligand-independent tyrosine phosphorylation.*

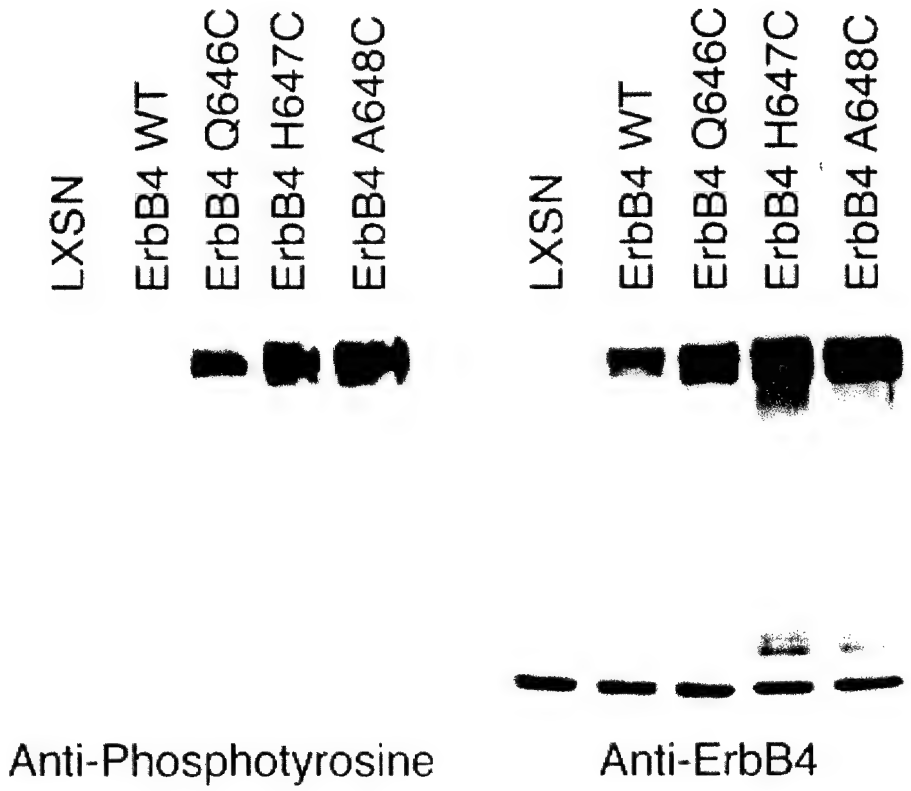



*Fig. 1.* ErbB4 mutants are constitutively tyrosine phosphorylated. ErbB4 expression and tyrosine phosphorylation were assayed in PA317 cells infected with retroviruses that direct the expression of wild-type ErbB4 or the ErbB4 mutants. Cells infected with the LXSN recombinant retrovirus vector control served as the negative control. Lysates were prepared from each of the cell lines, and ErbB4 was immunoprecipitated from 1000 $\mu$g of each lysate. Samples were resolved by SDS-PAGE, electroblotted to nitrocellulose, and immunoblotted with an anti-phosphotyrosine antibody (*left panel*). The blot was then stripped and probed with an anti-ErbB4 rabbit polyclonal antibody (*right panel*). The band at the *top* of the blots represents ErbB4.

From Penington, *et al.*, *Cell Growth Diff.* **13**: 247-256 (2002).

Figure 3. *Constitutively active ErbB4 mutants do not cause anchorage independence in FR3T3 fibroblasts.*

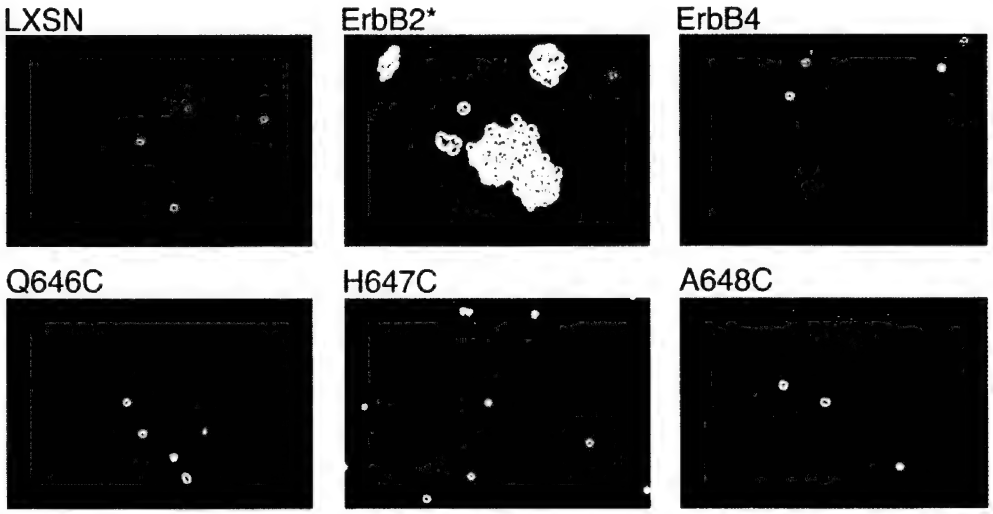
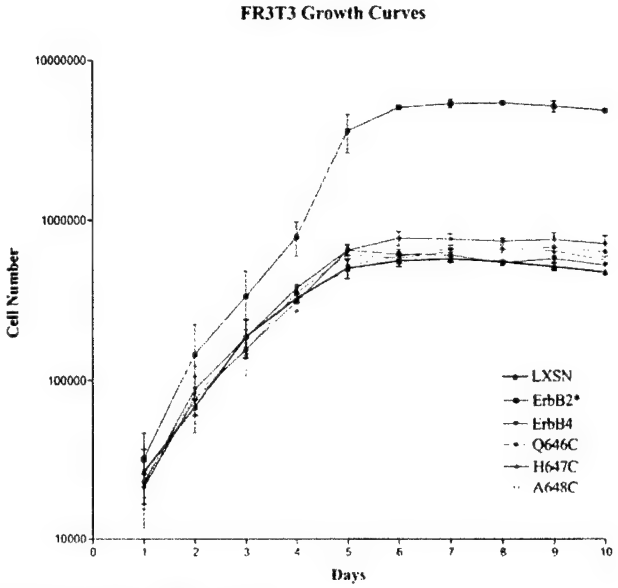



*Fig. 4.* Constitutively active ErbB4 receptors do not induce growth in semisolid medium. FR3T3 cells that stably express the LXSN vector control, the constitutively active ErbB2 mutant (ErbB2*), wild-type ErbB4, or the constitutively active ErbB4 mutants (Q646C, H647C, and A648C) were seeded in semisolid medium at a density of $2 \cdot 10^4$ cells/ml in 60-mm dishes. The cells were incubated for 10 days, after which images were recorded by photomicroscopy. Images shown are representative of those obtained in three independent experiments.

From Penington, *et al.*, *Cell Growth Diff.* **13**: 247-256 (2002).

Figure 4. *Constitutively active ErbB4 mutants do not cause an increase in growth rate or an increase in saturation density in fibroblasts.*

*Fig. 5.* Constitutively active ErbB4 mutants do not increase the growth rate of FR3T3 fibroblasts. FR3T3 cells that express the LXSN vector control, the constitutively active ErbB2* mutant, wild-type ErbB4, or the constitutively active ErbB4 mutants (Q646C, H647C, and A648C) were plated at a density of 2 $10^4$ cells in 60-mm dishes (700 cells/cm$^2$) and were incubated for 1–10 days. Cells were counted daily to assess growth rates and saturation densities. The means for three independent experiments; *bars*, SE.




From Penington, *et al.*, *Cell Growth Diff.* **13**: 247-256 (2002).

Figure 5. *Constitutively active ErbB4 mutants do not cause a loss of contact inhibition (focus formation) in FR3T3 fibroblasts.*

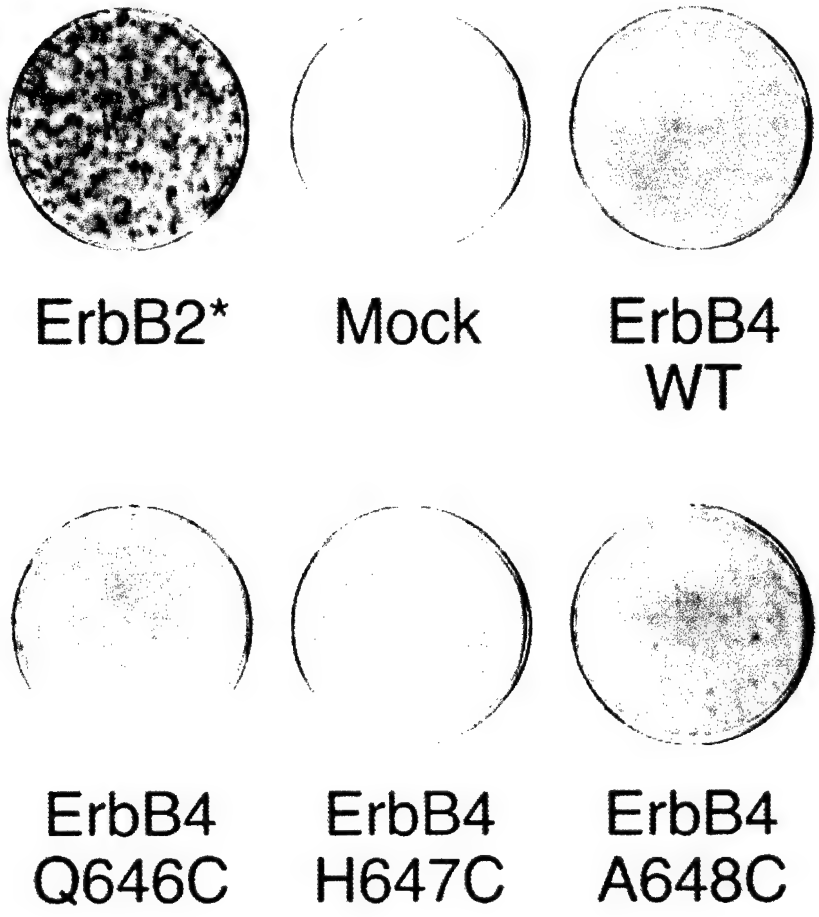
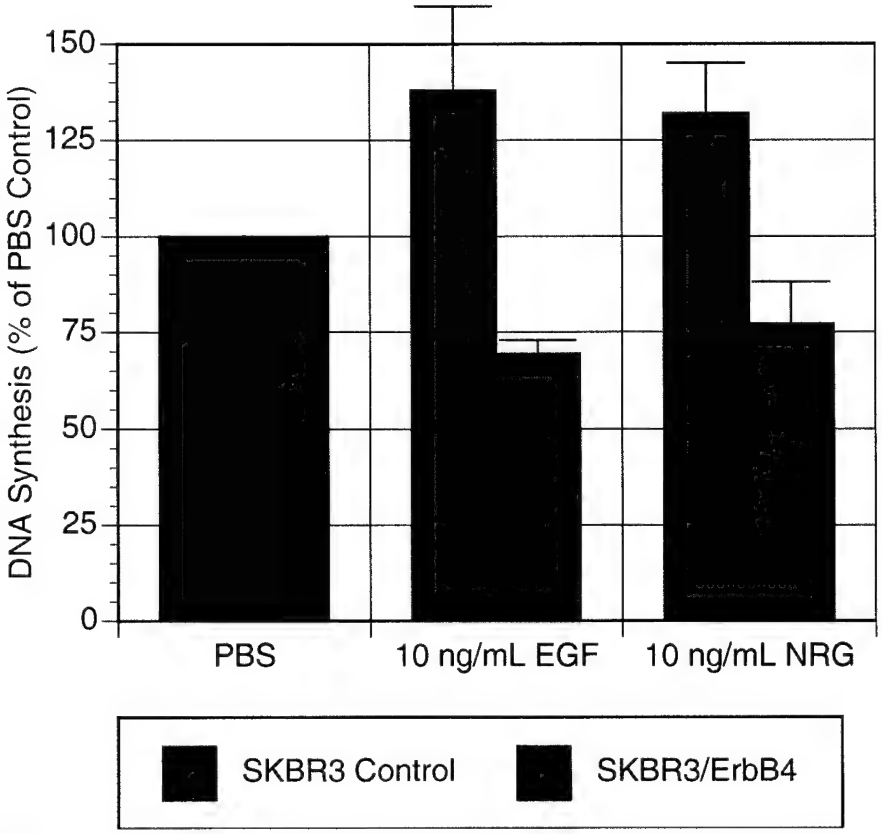



*Fig. 3.* Constitutively active ErbB4 receptors do not induce a loss of contact inhibition. FR3T3 fibroblasts infected with the LXSN (vector control) retrovirus, the wild-type ErbB4 retrovirus, the constitutively active ErbB2* retrovirus, or the constitutively active ErbB4 mutant retroviruses were assayed for loss of contact inhibition (focus formation).

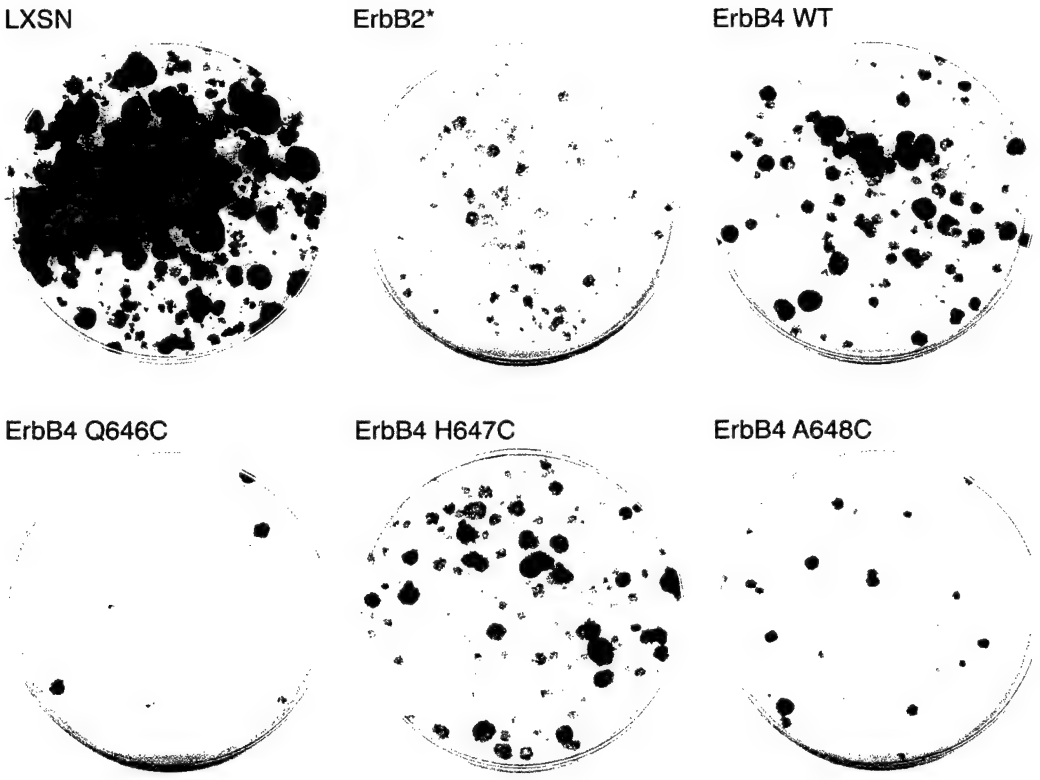
From Penington, *et al.*, *Cell Growth Diff.* **13**: 247-256 (2002).

Figure 6. *Overexpression of ErbB4 in the SKBR3 human breast tumor cell line causes inhibition of DNA synthesis by EGF and NRG.*



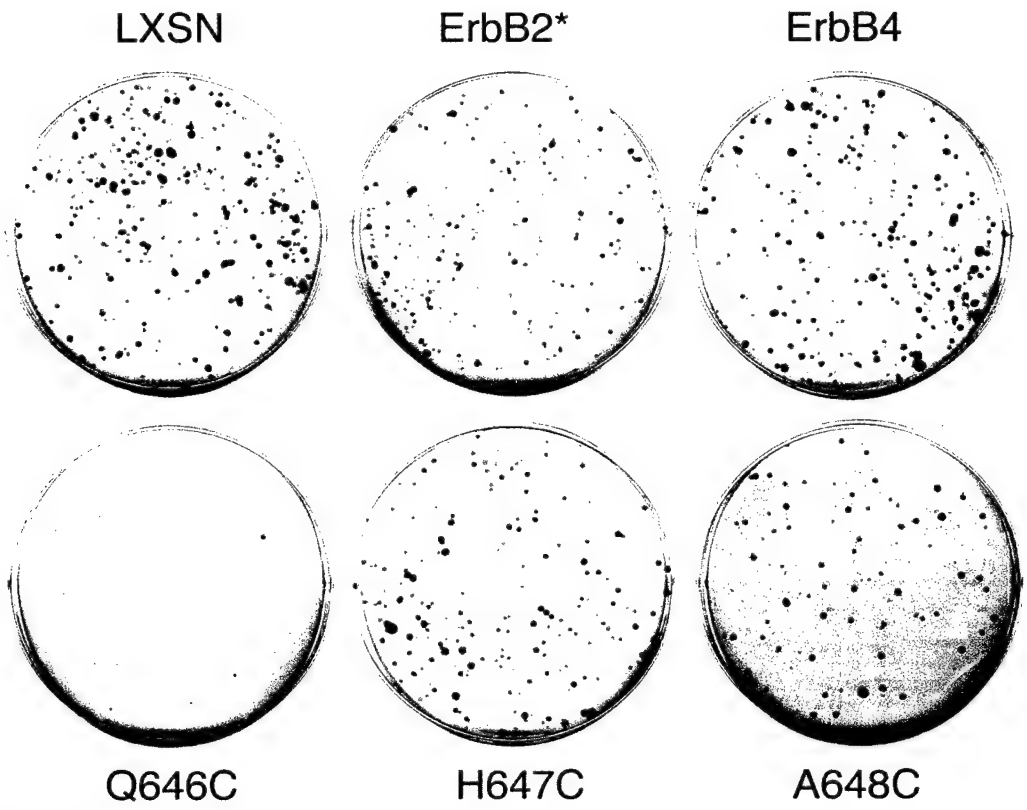

Unpublished data.

Figure 7. *The constitutively active Q646C ErbB4 mutant inhibits colony formation by the MCF10A human mammary epithelial cell line.*



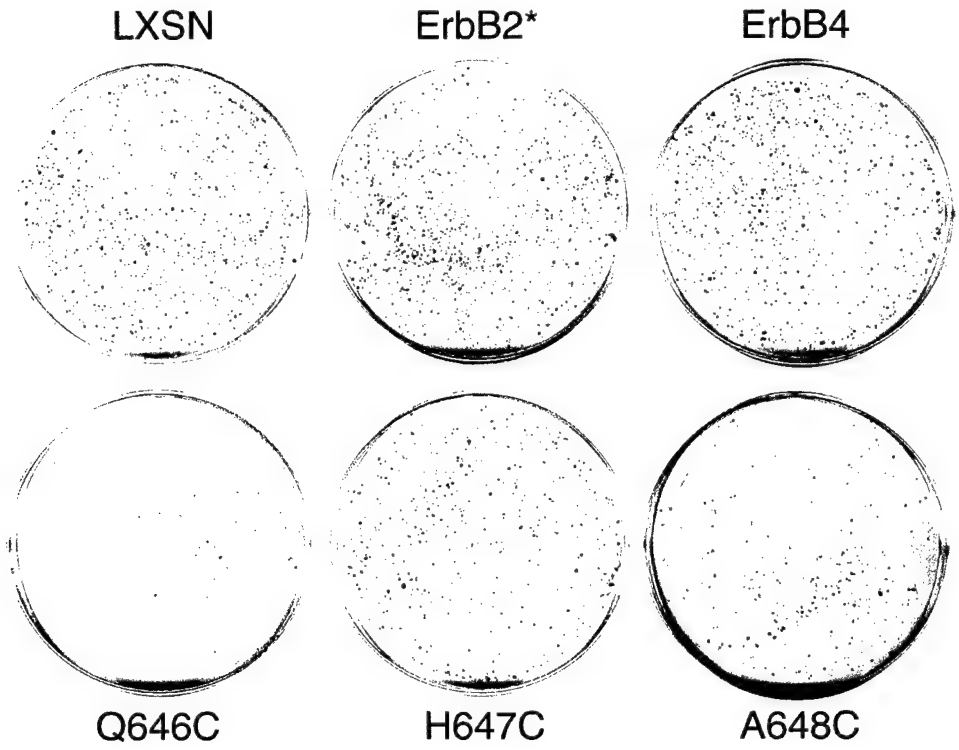

Unpublished data.

Figure 8. *The constitutively active Q646C ErbB4 mutant inhibits colony formation by the DU145 human prostate tumor cell line.*




Unpublished data.

Figure 9. *The constitutively active Q646C ErbB4 mutant inhibits colony formation by the PC-3 human prostate tumor cell line.*




Unpublished data.

Figure 10. *The constitutively active Q646C ErbB4 mutant specifically inhibits colony formation by the PC-3, DU-145, and LNCaP human prostate tumor cell lines.*

## Viral Titers (CFU/mL)

### C127

| plated | 7/11/01 | 7/25/01 | 10/4/01 | 11/7/01 | 11/30/01 | 7/3/02 | 7/10/02 | 7/12/02 |
|---|---|---|---|---|---|---|---|---|
| LXSN | 3.00E+05 | 4.10E+05 | 1.50E+05 | 2.00E+05 | | 5.24E+06 | 7.04E+05 | 6.24E+05 |
| ErbB2* | 6.30E+04 | 9.60E+04 | 2.60E+04 | 5.40E+04 | | 7.14E+05 | 3.71E+05 | 4.14E+05 |
| LXSN/ErbB4 | 6.50E+04 | 1.40E+05 | 2.30E+04 | 4.10E+04 | 5.30E+04 | 1.45E+05 | 1.90E+05 | 1.17E+05 |
| 646 | 7.60E+04 | 1.10E+05 | 2.10E+04 | 5.00E+04 | 4.60E+04 | 4.95E+05 | 6.92E+05 | 1.18E+06 |
| 647 | 8.50E+04 | 1.20E+05 | 2.90E+04 | 5.10E+04 | | 4.72E+05 | 7.51E+05 | 1.08E+06 |
| 648 | 1.50E+04 | 3.10E+04 | 1.70E+04 | 1.60E+04 | | 7.41E+04 | 1.41E+05 | 2.82E+05 |
| 646 Kin- | | | | 1.40E+04 | 1.10E+04 | | | |

### PC-3

| plated | 7/11/01 | 7/25/01 | 10/4/01 | 11/7/01 | 11/30/01 | 7/3/02 | 7/10/02 | 7/12/02 |
|---|---|---|---|---|---|---|---|---|
| LXSN | 1.10E+04 | 9.00E+04 | 4.00E+04 | 1.25E+05 | | 2.57E+05 | 2.24E+05 | 3.32E+04 |
| ErbB2* | 3.60E+03 | 1.10E+04 | 8.10E+03 | 2.30E+04 | | 3.64E+04 | 6.99E+04 | 3.18E+04 |
| LXSN/ErbB4 | 4.40E+03 | 1.70E+04 | 1.40E+04 | 2.60E+04 | 3.50E+04 | 1.53E+04 | 4.27E+04 | 9.23E+03 |
| 646 | 2.70E+02 | 9.70E+02 | 5.30E+02 | 2.10E+03 | 4.20E+03 | 1.60E+04 | 4.06E+04 | 1.15E+04 |
| 647 | 4.50E+03 | 1.90E+04 | 1.10E+04 | 3.40E+04 | | 3.89E+04 | 1.01E+05 | 8.06E+04 |
| 648 | 1.20E+03 | 5.60E+03 | 2.00E+03 | 5.90E+03 | | 1.28E+04 | 2.61E+04 | 1.72E+04 |
| 646 Kin- | | | | 9.70E+03 | 4.30E+04 | | | |

### DU-145

| plated | 7/11/01 | 7/25/01 | 10/4/01 | 11/7/01 | 11/30/01 | 7/3/02 | 7/10/02 | 7/12/02 |
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| ErbB2* | 4.70E+03 | 2.20E+04 | 4.20E+03 | 2.30E+03 | | 5.20E+04 | 6.85E+04 | 3.91E+04 |
| LXSN/ErbB4 | 4.90E+03 | 2.20E+04 | 8.70E+03 | 2.00E+03 | 7.60E+03 | 1.07E+04 | 4.08E+04 | 5.67E+03 |
| 646 | 1.30E+02 | 5.00E+02 | 1.30E+02 | 1.30E+02 | 6.00E+02 | 7.00E+03 | 4.13E+03 | 2.70E+03 |
| 647 | 4.50E+03 | 1.80E+04 | 2.70E+03 | 2.30E+03 | | 6.03E+04 | 3.43E+04 | 6.00E+04 |
| 648 | 1.30E+03 | 8.00E+03 | 2.50E+03 | 5.70E+02 | | 1.51E+04 | 1.29E+04 | 1.89E+04 |
| 646 Kin- | | | | 9.00E+02 | 3.70E+03 | | | |

### LNCaP

| plated | | | | | 11/30/01 | 7/3/02 | 7/10/02 | 7/12/02 |
|---|---|---|---|---|---|---|---|---|
| LXSN | | | | | | | | |
| ErbB2* | | | | | | | | |
| LXSN/ErbB4 | | | | | 2.10E+04 | | | |
| 646 | | | | | 2.00E+03 | | | |
| 647 | | | | | | | | |
| 648 | | | | | | | | |
| 646 Kin- | | | | | 1.90E+04 | | | |

## Viral Titer Ratios

### PC-3/C127 Ratio

| 7/11/01 | 7/25/01 | 10/4/01 | 11/7/01 | 11/30/01 | 7/3/02 | 7/10/02 | 7/12/02 | Average | Std. Error | n |
|---|---|---|---|---|---|---|---|---|---|---|
| 3.7 | 22.0 | 26.7 | 62.5 | | 4.9 | 31.8 | 5.3 | 22.4 | 8.0 | 7 |
| 5.7 | 11.5 | 31.2 | 42.6 | | 5.1 | 18.8 | 7.7 | 17.5 | 5.4 | 7 |
| 6.8 | 12.1 | 60.9 | 63.4 | 66.0 | 10.6 | 22.4 | 7.9 | 31.3 | 9.6 | 8 |
| 0.4 | 0.9 | 2.5 | 4.2 | 9.1 | 3.2 | 5.9 | 1.0 | 3.4 | 1.1 | 8 |
| 5.3 | 15.8 | 37.9 | 66.7 | | 8.2 | 13.5 | 7.5 | 22.1 | 8.5 | 7 |
| 8.0 | 18.1 | 11.8 | 36.9 | | 17.2 | 18.5 | 6.1 | 16.6 | 3.9 | 7 |
| | | | 69.3 | 390.9 | | | | 230.1 | 160.8 | 2 |

### DU145/C127 Ratio

| 7/11/01 | 7/25/01 | 10/4/01 | 11/7/01 | 11/30/01 | 7/3/02 | 7/10/02 | 7/12/02 | Average | Std. Error | n |
|---|---|---|---|---|---|---|---|---|---|---|
| 6.3 | 29.3 | 16.7 | 10.0 | | 4.7 | 18.6 | 3.1 | 12.7 | 3.5 | 7 |
| 7.5 | 22.9 | 16.2 | 4.3 | | 7.3 | 18.5 | 9.4 | 12.3 | 2.6 | 7 |
| 7.5 | 15.7 | 37.8 | 4.9 | 14.3 | 7.4 | 21.5 | 4.8 | 14.2 | 4.0 | 8 |
| 0.2 | 0.5 | 0.6 | 0.3 | 1.3 | 1.4 | 0.6 | 0.2 | 0.6 | 0.2 | 8 |
| 5.3 | 15.0 | 9.3 | 4.5 | | 12.8 | 4.6 | 5.6 | 8.1 | 1.6 | 7 |
| 8.7 | 25.8 | 14.7 | 3.6 | | 20.4 | 9.1 | 6.7 | 12.7 | 3.0 | 7 |
| | | | 6.4 | 33.6 | | | | 20.0 | 13.6 | 2 |

### LNCaP/C127 Ratio

| 11/30/01 | 7/3/02 | 7/10/02 | 7/12/02 | Average | Std. Error | n |
|---|---|---|---|---|---|---|
| | | | | #DIV/0! | #DIV/0! | 0 |
| | | | | #DIV/0! | #DIV/0! | 0 |
| 39.6 | | | | 39.6 | #DIV/0! | 1 |
| 4.3 | | | | 4.3 | #DIV/0! | 1 |
| | | | | #DIV/0! | #DIV/0! | 0 |
| | | | | #DIV/0! | #DIV/0! | 0 |
| 172.7 | | | | 172.7 | #DIV/0! | 1 |

Unpublished data.

# Constitutively Active ErbB4 and ErbB2 Mutants Exhibit Distinct Biological Activities[1]

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## Abstract

**ErbB4 is a member of the epidermal growth factor receptor (EGFR) family of tyrosine kinases, which includes EGFR/ErbB1, ErbB2/HER2/Neu, and ErbB3/HER3. These receptors play important roles both in normal development and in neoplasia. For example, deregulated signaling by ErbB1 and ErbB2 is observed in many human malignancies. In contrast, the roles that ErbB4 plays in tumorigenesis and normal biological processes have not been clearly defined. To identify the biological responses that are coupled to ErbB4, we have constructed three constitutively active ErbB4 mutants. Unlike a constitutively active ErbB2 mutant, the ErbB4 mutants are not coupled to increased cell proliferation, loss of contact inhibition, or anchorage independence in a rodent fibroblast cell line. This suggests that ErbB2 and ErbB4 may play distinct roles in tumorigenesis *in vivo*.**

## Introduction

ErbB4 (HER4/p180$^{erbB4}$) is a member of the EGFR[3] (EGFR/ErbB) family of receptor tyrosine kinases. These receptors play important roles in the embryonic development of heart, lung, and nervous tissues (1–4), and they have been implicated in the progression of metastatic disease. For example, EGFR/ErbB1 is overexpressed, amplified, or mutated in a number of human malignancies including breast, ovary, prostate, and lung cancers (5–7). ErbB2 overexpression correlates with tumor aggressiveness and poor prognosis in node-positive breast cancer patients (reviewed in Ref. 8). Finally, ErbB3 overexpression is observed in a subset of human mammary and gastric cancers (9, 10).

Some reports indicate that increased ErbB4 expression or signaling is associated with tumorigenesis. ErbB4 overexpression has been observed in a variety of cancers, including tumors of the thyroid, breast, and gastrointestinal tract (11–14). However, the prognostic significance of ErbB4 expression in tumors may also depend on which ErbB family members are coexpressed with ErbB4. In the case of childhood medulloblastoma (one of the most common solid tumors of childhood), patients with tumors overexpressing both ErbB2 and ErbB4 have a significantly worse prognosis than patients with tumors that overexpress either receptor alone (15).

Other reports indicate that increased ErbB4 expression or signaling correlates with tumor cell differentiation and reduced aggressiveness. ErbB4 overexpression in breast tumors is associated with progesterone receptor and estrogen receptor expression and a more favorable prognosis (16–17). In contrast, ErbB2 overexpression varies inversely with progesterone receptor and estrogen receptor levels and indicates tumors that are more likely to be metastatic and fatal (18). In one survey of common solid human cancers, the loss of ErbB4 expression is seen in a significant percentage of breast, prostate, and head and neck malignancies (19). These findings raise the intriguing possibility that ErbB4 is unique to the ErbB family of receptors in that ErbB4 expression and signaling may couple to reduced tumorigenesis or tumor cell proliferation. However, in the face of the conflicting evidence we have summarized here, it remains unclear what general or specific roles ErbB4 plays in differentiation, tumor suppression, or proliferation.

Efforts to elucidate ErbB4 function have been hampered by many factors. There are no known agonists or antagonists specific to the ErbB4 receptor. All of the peptide hormones of the EGF family that are capable of binding ErbB4 also bind at least one other ErbB family member. For example, epiregulin and betacellulin bind and activate both ErbB1 and ErbB4 (20, 21). Furthermore, ligands that do not bind an ErbB family receptor can still activate signaling by that receptor in *trans* through ligand-induced receptor heterodimerization (reviewed in Refs. 22, 23). For example, EGF stimulates ErbB2 tyrosine phosphorylation when ErbB2 is coexpressed with ErbB1, whereas EGF will not stimulate ErbB2 tyrosine phosphorylation in the absence of ErbB1 (24). Consequently, ligands that bind and directly activate ErbB4 (neuregulin, betacellulin, and epiregulin) also stimulate ErbB1, ErbB2, and ErbB3 signaling (Refs. 20, 21, 25, 26; reviewed in Refs. 22, 23). Therefore, in most contexts it is virtually impossible to use an EGF family hormone to study the functional consequences of ErbB4 signaling.

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[3] The abbreviations used are: EGFR, epidermal growth factor receptor; cfu, colony-forming unit(s); FR3T3, Fischer rat 3T3; LMP, low melting point; LTR, long terminal repeat.

To study ErbB4 function, we have opted to generate ErbB4 mutants that contain a cysteine substitution in the extracellular domain. This is predicted to result in constitutively dimerized and constitutively active ErbB4 mutants. Introducing cysteine residues to form covalently linked, dimeric, constitutively active receptor tyrosine kinases is not novel. This strategy has been used to generate dimeric, constitutively active mutants of EGFR/ErbB1 and ErbB2 (27, 28). Cysteine substitutions also lead to constitutively active mutants of the fibroblast growth factor receptors 2 and 3 (29, 30).

Here we report the generation and characterization of three constitutively active ErbB4 mutants. These mutants were generated through the introduction of a cysteine residue in the extracellular region of ErbB4. These mutants exhibit increased ligand-independent ErbB4 tyrosine phosphorylation, dimerization, and kinase activity. However, these constitutively active ErbB4 mutants do not induce increased proliferation, loss of contact inhibition, or anchorage-independent growth in FR3T3 fibroblasts. In contrast, a constitutively active ErbB2 mutant does induce increased proliferation, loss of contact inhibition, and anchorage-independent growth in FR3T3 fibroblasts. These results suggest that ErbB4 and ErbB2 couple to different signaling pathways and biological responses. These results also suggest that ErbB4 and ErbB2 may play distinct roles in tumorigenesis *in vivo*.

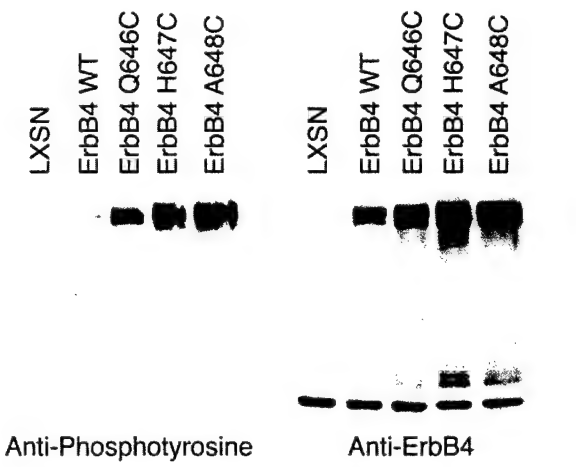


*Fig. 1.* ErbB4 mutants are constitutively tyrosine phosphorylated. ErbB4 expression and tyrosine phosphorylation were assayed in PA317 cells infected with retroviruses that direct the expression of wild-type ErbB4 or the ErbB4 mutants. Cells infected with the LXSN recombinant retrovirus vector control served as the negative control. Lysates were prepared from each of the cell lines, and ErbB4 was immunoprecipitated from 1000 $\mu$g of each lysate. Samples were resolved by SDS-PAGE, electroblotted to nitrocellulose, and immunoblotted with an anti-phosphotyrosine antibody (*left panel*). The blot was then stripped and probed with an anti-ErbB4 rabbit polyclonal antibody (*right panel*). The band at the *top* of the blots represents ErbB4.

## Results

**ErbB4 Mutants Are Constitutively Tyrosine Phosphorylated.** We substituted a single cysteine for amino acids Pro-645, Gln-646, His-647, Ala-648, and Arg-649 in the juxtamembrane region of the ErbB4 extracellular domain. These ErbB4 mutants (P645C, Q646C, H647C, A648C, and R649C) were generated in the context of the pLXSN-ErbB4 recombinant retroviral expression vector (26). Because these cysteine substitutions might cause inappropriate protein folding and decreased protein stability, we assayed the ErbB4 mutants for stable expression. We transfected the recombinant retroviral vectors containing the ErbB4 mutant constructs into the Ψ2 ecotropic retrovirus packaging cell line, selected for stable transformants, and generated pooled cell lines. We harvested low-titer ecotropic retrovirus stocks from these cell lines, and we analyzed the expression and tyrosine phosphorylation of the ErbB4 mutants in these cell lines. Three ErbB4 mutants (Q646C, H647C, and A648C) exhibit abundant expression and ligand-independent tyrosine phosphorylation (data not shown). However, the R649C ErbB4 mutant is not efficiently expressed, and the P645C mutant does not display ligand-independent tyrosine phosphorylation (data not shown).

Previous studies indicate that transfection and subsequent overexpression of ErbB family receptors lead to ligand-independent receptor tyrosine phosphorylation (31–33). Consequently, we were concerned that the ligand-independent phosphorylation of the Q646C, H647C, and A648C ErbB4 mutants in the transfected Ψ2 cells was a consequence of overexpression. Therefore, we infected the PA317 amphotropic retrovirus packaging cell line with the ErbB4 mutant recombinant ecotropic retroviruses at low multiplicities of infection (<0.1), selected for infected cells, and generated pooled cell lines. Because these cell lines were generated by infection at low multiplicities of infection, it is likely that each cell contains only one or two copies of the ErbB4 expression construct. This reduces the likelihood of ErbB4 overexpression in these cell lines.

We analyzed ErbB4 expression and tyrosine phosphorylation in the PA317 cell lines by anti-ErbB4 immunoprecipitation and either anti-ErbB4 (Fig. 1, *right panel*) or anti-phosphotyrosine (Fig. 1, *left panel*) immunoblotting. As expected, cells infected with the LXSN vector control retrovirus do not exhibit ErbB4 expression (Fig. 1, *right panel*) or tyrosine phosphorylation (Fig. 1, *left panel*). Cells infected with the wild-type or mutant ErbB4 retroviruses exhibit ErbB4 expression (Fig. 1, *right panel*). However, cells infected with the mutant ErbB4 retroviruses exhibit abundant ErbB4 tyrosine phosphorylation, whereas cells infected with the wild-type ErbB4 retrovirus exhibit minimal ErbB4 tyrosine phosphorylation (Fig. 1, *left panel*).

Quantification of the chemilumigrams shown in Fig. 1 suggests that the expression levels of the three ErbB4 mutants is less than three times greater than the amount of wild-type ErbB4 expression (Table 1). In contrast, the amounts of tyrosine phosphorylation of the three ErbB4 mutants appear to be much greater than the amount of wild-type ErbB4 tyrosine phosphorylation. Moreover, the ratios of ErbB4 tyrosine phosphorylation to ErbB4 expression for the three ErbB4 mutants appear to be at least four times greater than the ratio for wild-type ErbB4. These data suggest that the three ErbB4 mutants exhibit greater amounts of tyrosine phosphorylation on a per-molecule basis than does wild-type ErbB4. Consequently, these data indicate that the Q646C, H647C, and A648C ErbB4 mutants are constitutively active for signaling.

*Table 1* The Q646C, H647C, and A648C ErbB4 mutants exhibit increased normalized tyrosine phosphorylation

| Cell line | ErbB4 tyrosine phosphorylation | ErbB4 expression | Ratio |
|---|---|---|---|
| Wild-type ErbB4 | 210000 | 1800000 | 0.12 |
| ErbB4 Q646C | 1900000 | 3300000 | 0.58 |
| ErbB4 H647C | 2900000 | 4700000 | 0.62 |
| ErbB4 A648C | 4000000 | 4500000 | 0.89 |

**ErbB4 Mutants Have Increased *in Vitro* Kinase Activity.** Next, we assessed whether the increased tyrosine phosphorylation of the three ErbB4 mutants correlates with increased kinase activity. Equal amounts of the same lysates used for the experiments described in Fig. 1 were immunoprecipitated with an anti-ErbB4 polyclonal antibody. Kinase reactions were performed on the immunoprecipitates in the presence of [$\gamma$-$^{32}$P]ATP. The reaction products were resolved by SDS-PAGE on a 7.5% acrylamide gel. The gel was dried, and the reaction products were visualized by autoradiography.

In Fig. 2, we show that PA317 cells infected with the LXSN vector control retrovirus lack detectable ErbB4 kinase activity. Moreover, PA317 cells that express the three constitutively active ErbB4 mutants exhibit greater ErbB4 tyrosine kinase activity than cells that express wild-type ErbB4. Quantification of the bands on the autoradiogram indicates that the Q646C and H647C ErbB4 mutants exhibit approximately five times more kinase activity than does wild-type ErbB4, whereas the A648C ErbB4 mutant exhibits approximately nine times more kinase activity than does wild-type ErbB4. Given that the expression of the ErbB4 mutants (in these same lysates) is somewhat greater than the expression of wild-type ErbB4 (Fig. 1 and Table 1), it appears that the intrinsic kinase activity of the three ErbB4 mutants is three to four times greater than the intrinsic kinase activity of wild-type ErbB4.

**Constitutively Active ErbB4 Mutants Do Not Induce a Loss of Contact Inhibition.** Once we determined that the Q646C, H647C, and A648C ErbB4 mutants are constitutively active for signaling, we performed experiments using these mutants to identify the biological events coupled to ErbB4 signaling. A common assay for genes that encode growth control or signaling proteins involves introducing the gene into an established rodent fibroblast cell line and assaying for foci of piled-up cells. These foci indicate a loss of contact inhibition, a common attribute of malignant cells. Thus, this gene transfer assay is commonly used to identify genes that encode proteins that are coupled to malignant growth transformation.

Conflicting results have been obtained from assays for growth transformation by ErbB4. Transfection and consequent overexpression of ErbB4 induces foci (loss of contact inhibition) in NIH 3T3 clone 7 cells in the absence of ligand. Moreover, in these cells focus formation was stimulated by the ErbB4 ligand neuregulin 2$\beta$. In contrast, NIH 3T3 clone 7d cells (which lack EGFR expression) transfected with wild-type ErbB4 did not form foci in the presence or absence of neuregulin 1$\beta$; however, ErbB4 cotransfected with EGFR/ErbB1 or ErbB2 does induce foci in these cells (32, 33). One possible explanation is that ErbB4 lacks intrinsic transforming activity but does permit EGFR/ErbB1 or ErbB2 signaling and coupling to growth transformation in the presence of an ErbB4 ligand.

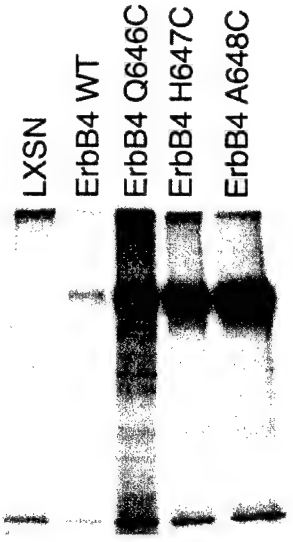


*Fig. 2.* Q646C, H647C, and A648C mutants exhibit increased *in vitro* kinase activity. Equal amounts of protein lysates (1000 $\mu$g) from PA317 cells that stably express wild-type ErbB4 or the ErbB4 mutants (Q646C, H647C, and A648C) were immunoprecipitated with an anti-ErbB4 rabbit polyclonal antibody. Lysates from PA317 cells that express the LXSN vector served as the negative control. Kinase reactions were performed on the immunoprecipitates in the presence of [$\gamma$-$^{32}$P]ATP. The products were resolved by SDS-PAGE. The gel was dried overnight and exposed to X-ray film for ~20 h to visualize the products of the kinase reactions.

To test whether ErbB4 signaling is sufficient to transform the growth of cultured rodent fibroblasts, FR3T3 fibroblasts were infected with 200 cfu of the ErbB4 mutant recombinant ecotropic retrovirus stocks and assayed for focus formation. Cells infected with 200 cfu of the LXSN vector control recombinant ecotropic retrovirus and with 200 cfu of the wild-type ErbB4 recombinant ecotropic retrovirus served as negative controls. Cells infected with 200 cfu of the constitutively active (V664E transmembrane domain) mutant ErbB2* retrovirus served as a positive control.

FR3T3 cells infected with the ErbB2* retrovirus had formed foci within 9 days after infection, whereas cells infected with the vector control retrovirus had not (Fig. 3). Furthermore, cells infected with the wild-type or mutant ErbB4 retroviruses had not formed foci within 9 days after infection. Within 18 days after infection, the foci arising from FR3T3 cells infected with the ErbB2* retrovirus had completely covered the surface of the tissue culture plate and had began to detach from the surface of the plate (data not shown). Within 18 days after infection, FR3T3 cells infected with the mutant ErbB4 retroviruses had formed relatively high-density clumps (data not shown). These high-density clumps did not exhibit the overlapping cell processes characteristic of foci (data not shown). The cells comprising these clumps were cloned and expanded into cell lines, as were cells from less dense regions of the cell monolayers. The cells from the clumps are morphologically indistinguishable from cells derived from the

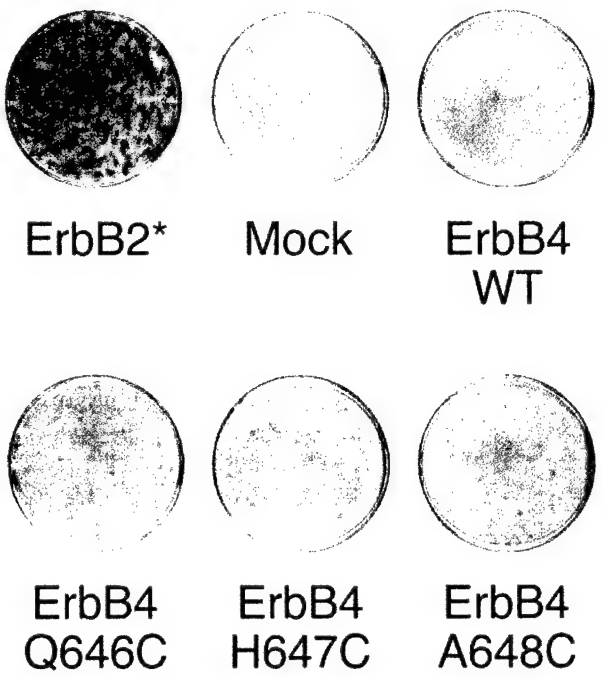


*Fig. 3.* Constitutively active ErbB4 receptors do not induce a loss of contact inhibition. FR3T3 fibroblasts infected with the LXSN (vector control) retrovirus, the wild-type ErbB4 retrovirus, the constitutively active ErbB2* retrovirus, or the constitutively active ErbB4 mutant retroviruses were assayed for loss of contact inhibition (focus formation).

less dense regions of the plates and are morphologically indistinguishable from cells that express wild-type ErbB4 or cells infected with the vector control retrovirus (data not shown). Again, this suggests that the constitutively active ErbB4 mutants do not transform the growth of FR3T3 fibroblasts.

We were concerned that the apparent failure of the constitutively active ErbB4 mutants to transform the growth of FR3T3 fibroblasts might be specific to this cell type. Consequently, we performed similar experiments with mouse C127 fibroblasts. Infection with the ErbB2* retrovirus resulted in numerous foci, whereas infection with the constitutively active ErbB4 mutant retroviruses did not (data not shown). Thus, again, whereas the constitutively active ErbB2* mutant readily induces foci in fibroblasts, the constitutively active ErbB4 mutants do not. This suggests that ErbB2 and ErbB4 couple to distinct cellular signaling pathways and biological events.

**Constitutively Active ErbB4 Mutants Do Not Induce Anchorage-independent Growth.** Next, we assayed FR3T3 cells that express the constitutively active ErbB4 mutants for growth while suspended in semisolid medium. Because anchorage-independent growth is another characteristic attribute of tumor cells *in vivo*, this assay is another way to determine whether ErbB4 signaling is coupled to malignant growth transformation.

FR3T3 cells were infected with the ErbB4 mutant recombinant ecotropic retroviruses at a low multiplicity of infection, and infected cells were selected using G418. Drug-resistant colonies of cells were pooled and expanded into cell lines. Control cell lines were generated through infection of FR3T3 cells with the wild-type ErbB4 retrovirus, the constitutively active ErbB2 retrovirus, and with the LXSN vector control retrovirus. These cell lines were seeded at a density of $2 \times 10^4$ cells/ml in 60-mm dishes in semisolid medium containing 0.3% LMP-agarose. Fresh medium containing LMP-agarose was added every 3 days. Photographs were taken of representative fields after 10 days.

FR3T3 cells that express the constitutively active ErbB2* mutant exhibit anchorage-independent growth (Fig. 4). In contrast, cells that were infected with the LXSN recombinant retroviral vector control and cells that express wild-type ErbB4 or the ErbB4 mutants do not exhibit anchorage-independent growth. The results of this assay are consistent with the results of the focus formation assay; both assays indicate that ErbB4 signaling is distinct from ErbB2 signaling in that ErbB4 signaling is not coupled to malignant growth transformation in FR3T3 fibroblasts.

**Constitutively Active ErbB4 Mutants Do Not Increase the Growth Rate or Saturation Density.** Another characteristic of malignantly transformed fibroblasts is that their growth rates and saturation densities are higher than those of their nontransformed counterparts. Indeed, constitutive ErbB2 signaling is coupled to increased growth rates (reviewed in Ref. 8). Thus, we assessed whether the constitutively active ErbB4 mutants affected the growth rate or saturation density of FR3T3 fibroblasts. The FR3T3 cell lines described earlier were seeded in 60-mm dishes at a density of $2 \times 10^4$ cells/dish (700 cells/cm$^2$). Cells were incubated for 10 days to permit proliferation. During this period, cells were counted every 24 h.

The growth rate of the cells that express ErbB2* is slightly greater than the growth rates of the other cell lines (Fig. 5). Note that the growth rates of the cells that express the constitutively active ErbB4 mutants are indistinguishable from the growth rates of cell lines that express wild-type ErbB4 or the vector control. The growth curves in Fig. 5 were used to determine the saturation densities for the six cell lines (Table 2). Note that the saturation density of the cell line that expresses ErbB2* is higher than the saturation densities of the other cell lines. Moreover, the saturation densities of the cell lines that express the ErbB4 mutants are not markedly higher than the saturation densities of the vector control cell line or the cell line that expresses wild-type ErbB4. Once again, these data suggest that constitutive ErbB4 signaling is not coupled to malignant growth transformation in fibroblasts. Thus, the signaling pathways and biological responses that are coupled to ErbB4 are distinct from those that are coupled to ErbB2.

**The Constitutively Active ErbB4 Mutants Are Expressed and Are Constitutively Tyrosine Phosphorylated in FR3T3 Cells.** We were concerned that the apparent failure of the constitutively active ErbB4 mutants to transform the growth of FR3T3 fibroblasts might be attributable to the absence of ErbB4 expression or constitutive tyrosine phosphorylation in these cells. In parallel with the infections described in Fig. 3, we infected FR3T3 cells with 200 cfu of the constitutively active mutant ErbB4 recombinant retroviruses

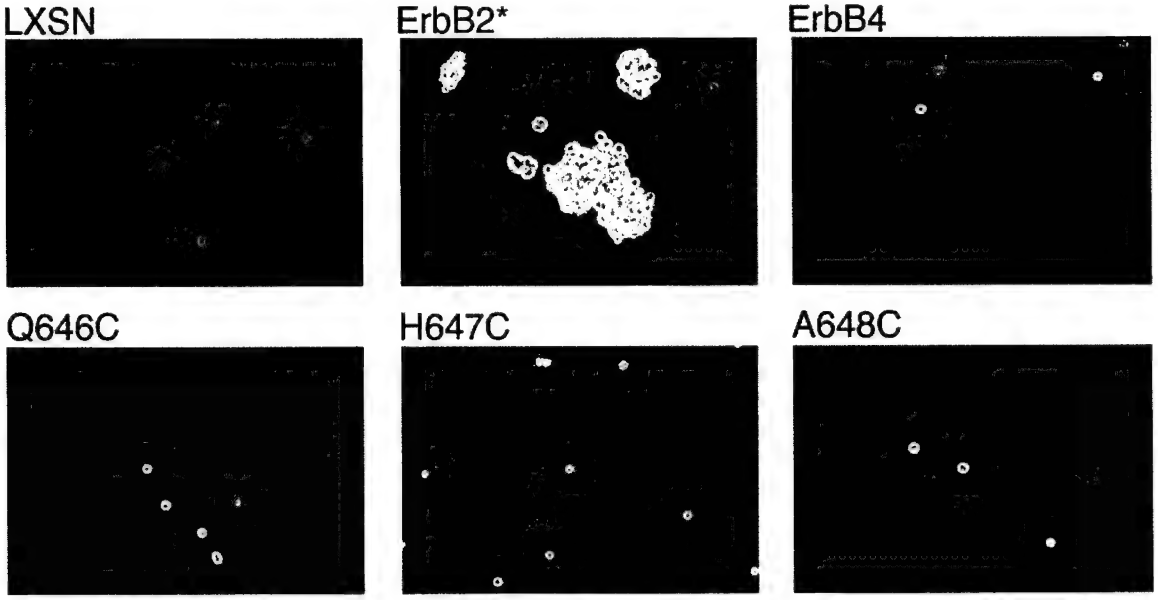


*Fig. 4.* Constitutively active ErbB4 receptors do not induce growth in semisolid medium. FR3T3 cells that stably express the LXSN vector control, the constitutively active ErbB2 mutant (ErbB2*), wild-type ErbB4, or the constitutively active ErbB4 mutants (Q646C, H647C, and A648C) were seeded in semisolid medium at a density of $2 \times 10^4$ cells/ml in 60-mm dishes. The cells were incubated for 10 days, after which images were recorded by photomicroscopy. Images shown are representative of those obtained in three independent experiments.

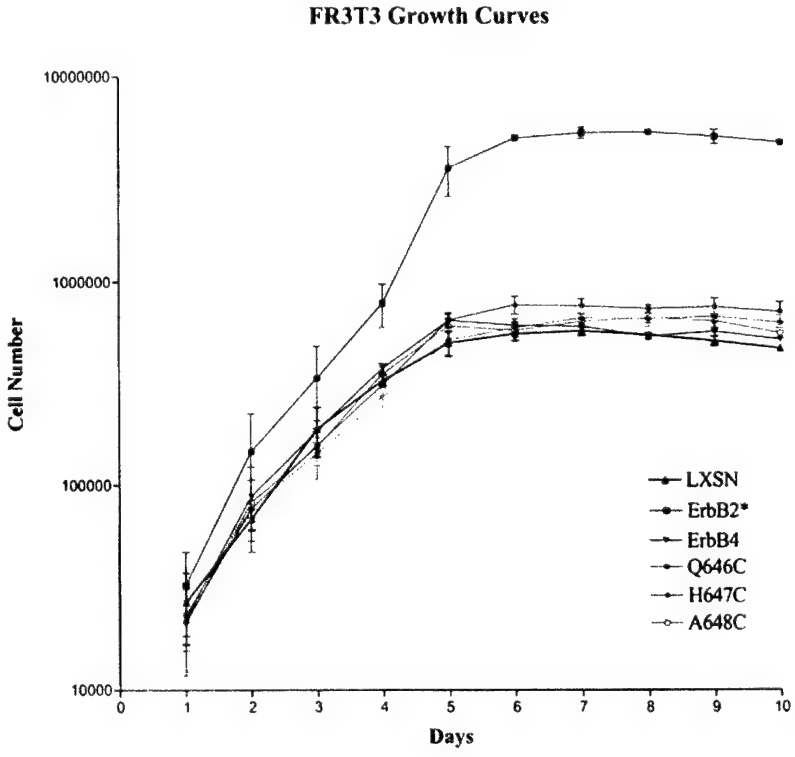


*Fig. 5.* Constitutively active ErbB4 mutants do not increase the growth rate of FR3T3 fibroblasts. FR3T3 cells that express the LXSN vector control, the constitutively active ErbB2* mutant, wild-type ErbB4, or the constitutively active ErbB4 mutants (Q646C, H647C, and A648C) were plated at a density of $2 \times 10^4$ cells in 60-mm dishes (700 cells/cm$^2$) and were incubated for 1–10 days. Cells were counted daily to assess growth rates and saturation densities. The means for three independent experiments; *bars,* SE.

and selected for stable infection using G418. As controls, we also infected FR3T3 cells with 200 cfu of the vector control retrovirus, 200 cfu of the ErbB2* retrovirus, and with 200 cfu of the wild-type ErbB4 retrovirus. Drug-resistant colonies were pooled and expanded into stable cell lines. The cell lines were starved of serum in the presence of 500 $\mu$M $Na_3VO_4$ (34) to decrease the background level of tyrosine phosphorylation and to increase the phosphorylation of the

*Table 2* Constitutively active ErbB4 mutants do not increase the saturation density of FR3T3 fibroblasts

| | Saturation Densities |
|---|---|
| LXSN | $5.8 \pm 0.3 \times 10^5$ |
| ErbB2* | $5.4 \pm 0.1 \times 10^6$ |
| ErbB4 | $6.1 \pm 0.5 \times 10^5$ |
| Q646C | $6.6 \pm 0.6 \times 10^5$ |
| H647C | $7.6 \pm 0.7 \times 10^5$ |
| A648C | $6.6 \pm 0.4 \times 10^5$ |

constitutively active ErbB4 mutants. We prepared lysates and analyzed ErbB4 expression and tyrosine phosphorylation by precipitation with an anti-ErbB4 antibody and sequential anti-phosphotyrosine and anti-ErbB4 immunoblotting.

In Fig. 6, *lower panel,* we show that ErbB4 expression is detectable in the FR3T3 cell lines infected with the wild-type ErbB4 retrovirus or the constitutively active ErbB4 mutant retroviruses. However, ErbB4 tyrosine phosphorylation is observed only in the FR3T3 cell lines infected with the constitutively active ErbB4 mutant retroviruses (Fig. 6, *upper panel*). The amount of phosphorylation exhibited by the ErbB4 mutants is less than the amount of phosphorylation exhibited by the constitutively active ErbB2 mutant. Furthermore, the expression of wild-type ErbB4 appears to be less than the expression of the ErbB4 mutants. Nonetheless, these data suggest that the apparent failure of the constitutively active ErbB4 mutants to transform the growth of FR3T3 fibroblasts is not attributable to an absence of expression and tyrosine phosphorylation of these mutants in these cells.

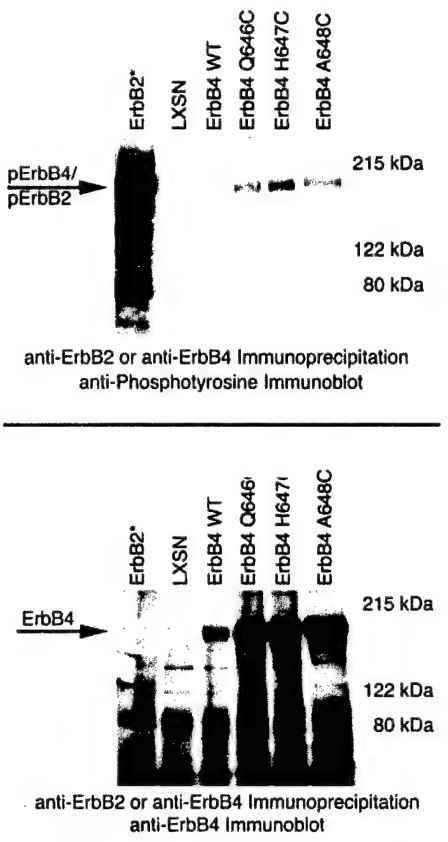


*Fig. 6.* Constitutively active ErbB4 mutants are expressed and are constitutively tyrosine phosphorylated in FR3T3 cells. ErbB4 expression and tyrosine phosphorylation were assayed in FR3T3 cells infected with retroviruses that direct the expression of wild-type ErbB4 or the ErbB4 mutants. Cells infected with the LXSN recombinant retrovirus vector control or with the ErbB2* retrovirus served as controls. Lysates were prepared from each of the cell lines, and ErbB receptors were precipitated from 1.5 mg of each lysate using protein A-Sepharose and either an anti-ErbB4 rabbit polyclonal antibody or an anti-ErbB2 rabbit polyclonal antibody. Samples were resolved by SDS-PAGE, electroblotted to nitrocellulose, and immunoblotted with an anti-phosphotyrosine antibody (*upper panel*). The blot was then stripped and probed with an anti-ErbB4 rabbit polyclonal antibody (*lower panel*). *Arrows,* positions of ErbB2 and ErbB4 on the blots.

## Discussion

In this report, we describe the construction and initial characterization of three constitutively active ErbB4 mutants. These mutants display increased dimerization (data not shown) and ligand-independent tyrosine phosphorylation and kinase activity. In these respects, the ErbB4 mutants resemble constitutively active mutants of ErbB2 or EGFR. However, unlike constitutively active ErbB2 mutants, these mutants are not coupled to malignant growth transformation in FR3T3 fibroblasts; they do not induce foci, anchorage-independent growth, or increases in the growth rate or saturation density. These data suggest that ErbB2 and ErbB4 play distinct roles in tumorigenesis *in vivo*. This conclusion is supported by the observation that NIH3T3 clone 7d cells do not form foci after ErbB4 transfection and treatment with the ErbB4 ligand neuregulin but do form foci after ErbB2 and ErbB4 cotransfection and neuregulin treatment (32, 33).

Of course, another potential explanation is that the amounts of tyrosine phosphorylation displayed by the three constitutively active ErbB4 mutants are insufficient to couple to malignant growth transformation in fibroblasts. This is consistent with the observation that the three constitutively active ErbB4 mutants are less phosphorylated than the constitutively active ErbB2 mutant (Fig. 6). However, anti-phosphotyrosine immunoblotting is not a sensitive method for assessing ErbB family receptor signaling and coupling to biological responses. Indeed, the neuregulin concentration required for maximal ErbB4 tyrosine phosphorylation is ~10-fold greater than the neuregulin concentration sufficient for maximal ErbB family receptor coupling to biological responses. Furthermore, the neuregulin concentration sufficient for maximal ErbB family receptor coupling to biological responses stimulates, at most, only modest amounts of ErbB4 tyrosine phosphorylation (26). Thus, it is not likely that the failure of the constitutively active ErbB4 mutants to couple to malignant growth transformation in fibroblasts is attributable to insufficient ErbB4 tyrosine phosphorylation.

Clearly, additional work is necessary to define the roles that ErbB4 plays in tumorigenesis and in regulating cellular functions *in vivo*. However, important clues have emerged to guide these future studies. In a significant percentage of breast tumor samples, ErbB4 expression correlates with estrogen receptor expression, which indicates a favorable prognosis (16–17). Furthermore, ErbB4 expression is fre-

quently lost in tumors of the breast and prostate (19). Finally, ligands for ErbB4 can induce terminal differentiation and growth arrest of some mammary tumor cell lines (35–37). These data indicate that ErbB4 signaling may be coupled to differentiation, growth arrest, and tumor suppression. The ErbB4 mutants described in this study will enable us to evaluate this hypothesis. Indeed, preliminary data from our laboratory indicate that the Q646C ErbB4 mutant causes reduced colony formation in plastic dishes by a number of cultured human breast and prostate tumor cell lines.

We will also perform additional studies to characterize the biochemistry of signaling by the three ErbB4 mutants. Whereas these mutants exhibit greater ligand-independent tyrosine phosphorylation and autokinase activity than the wild-type receptor, it is unclear whether this is attributable to increased intrinsic kinase activity or attributable to increased availability of the substrate. Additional experiments are warranted to distinguish between these two possibilities.

Another area of future study will focus on identifying the mechanisms by which ErbB4 is coupled to biological responses. Initial studies will identify the sites of ErbB4 tyrosine phosphorylation for these mutants. If our preliminary studies indicating that the Q646C ErbB4 mutant is coupled to prostate and mammary tumor cell growth arrest hold true, then we will use genetic strategies to identify the sites of ErbB4 tyrosine phosphorylation that are sufficient and necessary to couple the Q646C ErbB4 mutant to this biological response. A similar strategy has been used to identify the sites of ErbB2 and platelet-derived growth factor receptor tyrosine phosphorylation that are critical for coupling these receptors to biological responses (38, 39).

Once we have identified the site(s) of tyrosine phosphorylation that is sufficient for coupling to biological responses, we will identify signaling proteins that bind this phosphorylation site and couple it to biological responses. Using this strategy, we will begin to characterize the ErbB4 signaling pathway. Our prediction is that the three constitutively active ErbB4 mutants are phosphorylated on different tyrosine residues and that these mutants differentially couple to biological responses. We have shown previously that different ErbB4 ligands cause phosphorylation on different sites on ErbB4 and differential coupling to biological responses (40). Moreover, one cysteine substitution mutation in the rat ErbB2 extracellular domain (V656C) results in low amounts of constitutive receptor tyrosine phosphorylation and efficient coupling to malignant growth transformation in rodent fibroblasts. In contrast, another rat ErbB2 extracellular domain cysteine substitution mutant (T657C) exhibits very high levels of constitutive receptor tyrosine phosphorylation but a relatively low amount of coupling to malignant growth transformation in rodent fibroblasts (28).

We were somewhat surprised to discover that the three constitutively active ErbB4 mutants failed to couple to malignant growth transformation in a rodent fibroblast cell line. Nonetheless, these mutants will enable us to assess ErbB4 function in a wide variety of cell, tissue, and organismal contexts. Given that ErbB4 appears to regulate diverse functions in a number of distinct contexts, much work remains to complete this story.

## Materials and Methods

**Cell Lines, Cell Culture, and Antibodies.** The Ψ2, PA317, C127, and FR3T3 cell lines were generous gifts from Daniel DiMaio (Yale University New Haven, CT). All cell lines were propagated in DMEM supplemented with 10% FBS, 50 IU/ml penicillin, 50 μg/ml streptomycin (Mediatech), and 0.25 μg/ml Fungizone (Amphotericin B; Life Technologies, Inc.). Recombinant cell lines generated in the course of the experiments described in this report were propagated in the medium described above supplemented with 200 μg/ml G418 (Mediatech).

The anti-ErbB4 mouse monoclonal (SC-8050), anti-ErbB4 rabbit polyclonal (SC-283), and anti-ErbB2 rabbit polyclonal (C-18) antibodies were purchased from Santa Cruz Biotechnology. Goat antimouse and goat antirabbit horseradish peroxidase-conjugated antibodies were purchased from Pierce. Enhanced chemiluminescence (ECL) Western blotting reagents, Redivue adenosine 5′-[$\gamma$-$^{32}$P]triphosphate, and Protein-A Sepharose (CL-4B) were purchased from Amersham Pharmacia Biotech. The 4G10 anti-phosphotyrosine mouse monoclonal antibody was purchased from Upstate Biotechnology.

**Plasmids.** The recombinant retroviral vector pLXSN (41) was obtained from Daniel DiMaio (Yale University). This construct contains two recombinant LTRs derived from the Maloney murine leukemia virus and the Maloney murine sarcoma virus. These LTRs flank the Ψ packaging signal and the aminoglycoside 3′-phosphotransferase (*Neo*$^R$) gene under the transcriptional control of the SV40 early promoter. The *Neo*$^R$ gene confers resistance to the aminoglycoside antibiotic G418 (geneticin; Life Technologies, Inc.).

The recombinant retroviral construct pLXSN-ErbB4 (26) was generated by subcloning the human ErbB4 cDNA into pLXSN. In this construct, the ErbB4 cDNA is under the transcriptional control of the upstream LTR. The recombinant retroviral construct pLXSN-ErbB2* (42) was a gift of Lisa Petti (Albany Medical College, Albany, NY). It was generated by subcloning the cDNA encoding the constitutively active rat ErbB2 mutant (V664E transmembrane domain mutant, ErbB2*) into pLXSN. In this construct, the ErbB2* cDNA is under the transcriptional control of the upstream LTR.

**ErbB4 Mutagenesis.** The plasmid pLXSN-ErbB4 was used as the template for site-directed mutagenesis (QuikChange Site Directed Mutagenesis kit; Stratagene) to construct the putative constitutively active ErbB4 mutants. The mutants were constructed by introducing mutations that substitute a cysteine residue for proline 645, glutamine 646, histidine 647, alanine 648, or arginine 649 in the ErbB4 extracellular juxtamembrane domain. These mutants are denoted as follows: P645C, Q646C, H647C, A648C, and R649C. A new restriction enzyme site was also engineered in each mutant to facilitate the identification of the mutants. The following primers were used for mutagenesis. "T" denotes the upper primer, whereas "B" denotes the lower primer. The novel cysteine codons and anticodons are indicated by bold type, the point mutations that create the novel cysteine residues are double underlined, and the novel restriction enzyme sites are singly underlined.

P645CT:5′-ATTTACTACCCATGGACCGGTCATTCCACTT
TATGCCAACATGCTAGAACTCCC-3′
P645CB:5′-GGGAGTTCTAGCATGTTGGCATAAAGTGGA
ATGACCGGTCCATGGGTAGTAAAT-3′
Q646CT:5′-TACTACCCATGGACCGGTCATTCCACTTTAC
CATGCCATGCTAGAACTCCCCTG-3′
Q646CB:5′-CAGGGGAGTTCTAGCATGGCATGGTAAAGT
GGAATGACCGGTCCATGGGTAGTA-3′
H647CT:5′-CATTTACTACCCATGGACCGGTCATTCCACT
TTACCACAATGTGCTAGAACTCCCCT-3′
H647CB:5′-AGGGGAGTTCTAGCACATTGTGGTAAAGTG
GAATGACCGGTCCATGGGTAGTAAATG-3′
A648CT:5′-TCCACTTTACCACAACATTGTAGAACTCCTC
TGATTGCAGCTGGA-3′
A648CB:5′-TCCAGCTGCAATCAGAGGAGTTCTACAATG
TTGTGGTAAAGTGGA-3′
R649CT:5′-ACTTTACCACAACATGCTTGCACTCCTCTGA
TTGCAGCTGGA-3′
R649CB:5′-TCCAGCTGCAATCAGAGGAGTGCAAGCATG
TTGTGGTAAAGT-3′

The site-directed mutagenesis reactions were performed according to the manufacturer's instructions. Standard techniques (43) were used for bacterial transformations, small-scale plasmid DNA preparations, restriction enzyme analysis of the clones, and large-scale plasmid DNA preparations. Positive clones were sequenced by the University of Wisconsin-Madison Biotechnology Center to confirm their identity.

**Production of Recombinant Retroviruses and Retroviral Infections.** The ErbB4 mutant constructs were transfected using standard techniques (44, 45) into the $\psi 2$ ecotropic retrovirus packaging cell line (46) to generate cell lines that express the ErbB4 mutants and to package the constructs into low-titer ecotropic retrovirus particles (44, 45). $\psi 2$ cells were transfected with the pLXSN vector control plasmid, pLXSN-ErbB4, and pLXSN-ErbB2* to generate control cell lines and recombinant ecotropic retroviruses. The PA317 amphotropic packaging cell line (47) and the FR3T3 rat fibroblast cell line were infected with the ecotropic recombinant retroviruses using standard techniques (44, 45) to generate additional cell lines that express the ErbB4 mutants.

**Immunoblot Assays for Receptor Tyrosine Phosphorylation and Expression.** The analysis of ErbB4 and ErbB2 tyrosine phosphorylation by immunoprecipitation and anti-phosphotyrosine immunoblotting has been described previously (21, 26). Briefly, cell lysates were generated, and protein content was quantified using a Coomassie Protein Assay Reagent (Ref. 48; Pierce Chemical). ErbB2 or ErbB4 was immunoprecipitated from equal amounts of protein using specific antibodies. The immunoprecipitates were resolved by SDS-PAGE on a 7.5% acrylamide gel and were electrotransferred onto nitrocellulose. The blots were probed with the anti-phosphotyrosine monoclonal antibody 4G10. Antibody binding was detected and visualized using a goat antimouse horseradish peroxidase-coupled antibody and enhanced chemiluminescence. The blots were then stripped and probed with the anti-ErbB4 polyclonal antibody to assess ErbB4 expression levels. Antibody binding was detected and visualized using a goat antimouse horseradish peroxidase-coupled antibody and enhanced chemiluminescence.

The amounts of receptor tyrosine phosphorylation and expression were quantified by digitizing the chemilumigrams using a Linotype-Hell Jade two-dimensional scanning densitometer set at 600-dpi resolution. The bands on the images were quantified using NIH Image for Macintosh v1.6 software. Values are expressed as arbitrary units. Background levels were computed using the vector control lanes and were subtracted from the gross values to produce net receptor expression and tyrosine phosphorylation values. The digitized images were also cropped and annotated using Adobe Photoshop for Macintosh v3.0.5 software.

***In Vitro* Kinase Assay.** ErbB2 and ErbB4 were immunoprecipitated from protein extracts from PA317 cells as described previously (26). Immune complex kinase reactions were performed as described previously (31). Briefly, 35 $\mu$l of protein A-Sepharose and 5 $\mu$l of anti-ErbB2 or anti-ErbB4 rabbit polyclonal antibodies were used to immunoprecipitate the receptors from lysates containing the same amount of protein (1000 $\mu$g). Immunoprecipitates were washed five times in 500 $\mu$l of kinase buffer [20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, and 3 mM $MnCl_2$]. After the last wash, the samples were resuspended in 100 $\mu$l of kinase buffer supplemented with 10 $\mu$Ci of [$\gamma$-$^{32}$P]ATP and were incubated for 10 min at room temperature to permit the kinase reaction to occur. The beads were then washed two times in NET-N buffer (49) and boiled for 5 min in SDS-PAGE protein sample buffer. The samples were resolved by SDS-PAGE on a 7.5% acrylamide gel. The gels were dried overnight and exposed to X-ray film for ~20 h. The autoradiograms were digitized using a Linotype-Hell Jade two-dimensional scanning densitometer set at 600-dpi resolution. The bands on the images were quantified using NIH Image for Macintosh v1.6 software. Values are expressed as arbitrary units. Background levels were computed using the vector control lanes and were subtracted from the gross values to produce net kinase activity values. The digitized images were also cropped and annotated using Adobe Photoshop for Macintosh v3.0.5 software.

**Focus Formation Assay for Loss of Contact Inhibition.** FR3T3 and C127 cells were infected with recombinant ecotropic retroviruses as described earlier and in reports published previously (44, 45). Briefly, 60-mm dishes of cells at ~70% confluence were infected with ecotropic retrovirus stocks. Approximately 24 h after infection, cells were passaged into three 60-mm dishes. Cells were maintained in DMEM supplemented with 10% FBS until foci appeared. During this period, the medium was changed every 3 days. Once robust foci appeared, cells were fixed in 100% methanol and stained with Giemsa (Fisher) to visualize the foci. The plates were digitized using a Linotype-Hell Jade two-dimensional scanning densitometer set at 600-dpi resolution. The digitized images were cropped and annotated using Adobe Photoshop for Macintosh v3.0.5 software.

**Assay for Anchorage Independence.** FR3T3 cells were seeded at a density of $2 \times 10^4$ cells in 60-mm dishes containing 2.5 ml of 0.3% LMP-agarose (Life Technologies, Inc.)

as described previously (50). Every 3 days, DMEM supplemented with 10% FBS and 0.3% LMP-agarose was added to each plate. The cells were incubated at 37°C for 10 days, and fields were photographed with an Olympus OM-10 camera attached to an Olympus CK-2 phase-contrast inverted microscope. The images were digitized by the photofinisher. These images were cropped and annotated using Adobe Photoshop for Macintosh v3.0.5 software. Images are representative of three independent experiments.

**Growth Rate/Saturation Density Assay.** Stable FR3T3 cell lines expressing the wild-type ErbB4 receptor, ErbB2*, or the ErbB4 mutants (Q646C, H647C, and A648C) were plated in 10 60-mm dishes at a density of $2 \times 10^4$ cells/dish. Cells were incubated from 1 to 10 days at 37°C. Cells were counted (Coulter Counter ZM) each day for a total of 10 days. The mean and SE are representative of three independent experiments.

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